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Suppression of basement membrane type IV collagen degradation and cell invasion in human melanoma cells expressing an antisense RNA for MMP-1

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Abstract

During progression from benign nevus to vertical growth phase melanoma, melanocytes acquire the ability to invade into the dermis. This process requires rupture of the basal lamina and dissolution of dermal type I collagen. Metastases-derived human melanoma MIM cells have an invasive ability in vitro which is dependent on metalloproteinases. In the present study we analysed the role of type I collagenase (MMP-1) in melanoma invasion using MIM cells in which the constitutive expression of MMP-1 was suppressed by stable transfection with a plasmid vector expressing a 777 bp antisense fragment of MMP-1 genomic DNA. Two clones were isolated in which MMP-1 mRNA expression was blocked by 90–96% with a corresponding loss in protein synthesis. In their morphological appearance and growth rate in vitro these cells were indistinguishable from wild type cells or control cells transfected with the same vector expressing the MMP-1 fragment in the sense orientation. Their mRNA and protein levels for type IV collagenase (MMP-2) were unchanged as assessed by Northern and Western blot analyses and by gelatin zymography. However, when the invasive ability of the cells was measured, we found that in addition to type I collagen, invasion through type IV collagen and a reconstituted, type IV collagen-containing basement membrane (Matrigel) were also significantly inhibited as compared to normal or sense-transfected cells. The results indicate that despite the presence of functional MMP-2, degradation of type IV collagen matrices by the melanoma cells was dependent on expression of MMP-1.

Keywords: Invasion; Interstitial collagenase; Metalloproteinase; Metastasis

1. Introduction

Cellular invasion is required for many physiological processes both normal (e.g., matrix remodelling) and pathological (e.g., tumor metastasis) [1]. Metastasis is a complex multistep process during which tumor cells invade through different extracellular matrices (ECM) such as basement membrane and connective tissue and give rise to new foci at sites distant from the primary tumor.

The ECM is a complex structure composed of collagen, glycoproteins and proteoglycans forming a dense meshwork normally impenetrable to migrating cells [2–4]. Tumor cells can invade the ECM through the upregulated expression and release of hydrolytic enzymes which can solubilize this matrix [1,5]. The

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enzymes mediating ECM degradation belong to several known classes of proteinases including the metalloproteinases (MMPs) and the serine, cysteine and aspartic proteinases [1,2]. Among the metalloproteinases, several collagenases have been identified including interstitial collagenase (MMP-1) which degrades types I, II and III collagen and two type IV collagenases; a 72 kDa gelatinase designated MMP-2 and a 92 kDa gelatinase designated MMP-9 which can degrade types IV and V and denatured collagen [2,6]. Another metalloproteinase implicated in metastasis is stromelysin-1 (MMP-3) which can degrade fibronectin, laminin, proteoglycans and type IV collagen [7].

The MMPs have similar primary structures [8,9] and are secreted in a zymogen form which can be activated by a 'cysteine switch' mechanism resulting in autolytic cleavage at the N-terminus of the proenzyme [10]. Their extracellular activity is regulated by endogenous tissue inhibitors of metalloproteinases of which three TIMP-1, TIMP-2 and TIMP-3 have been identified to date [11]. The net proteolytic activity associated with the cells reflects therefore the balance between the activities of the enzymes and their inhibitors [12,13]. MMPs have been implicated in the invasion and metastasis of different malignancies [14–16] including melanoma [17–19]. Much of the available evidence implicates MMP-2 and MMP-9 in tumor invasion [20–22]. Little is presently known, however, about the contribution of MMP-1 to this process [16,18].

In the present study we assessed the invasive ability of human melanoma cells in which MMP-1 expression was suppressed by stable transfection with a plasmid vector expressing MMP-1 DNA in the antisense orientation. Cell invasion was measured using filters coated with reconstituted basement membrane (Matrigel), type I and type IV collagen. Our evidence indicates that the invasiveness of these cells through all three matrices required MMP-1 expression.

2. Materials and methods

2.1. Cell lines

The melanoma MIM line was established from an inguinal lymph node metastasis of a male melanoma patient, as we previously described [23]. Cells were grown as a monolayer culture in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FCS), 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). Once weekly the monolayers were dispersed with a 0.5 mM EDTA solution and the cells reseeded at a dilution of 1:5.

2.2. Plasmid construction and transfection

A 777 bp genomic DNA fragment of MMP-1 was generated by the polymerase chain reaction (PCR) performed on a crude lysate of normal lung fibroblasts. The reaction ran for 30 cycles in an automated thermal cycler (Perkin-Elmer/Cetus) using a 60°C annealing temperature and standard conditions for denaturation and extension. The primers corresponding to bases 1–25 (primer 1) and 246–278 (primer 2) of the human collagenase cDNA [24] were synthesized using the Applied Biosystem DNA Synthesizer. The PCR product spanning the entire noncoding sequence, exon 1, intron 1 and a 36 bp fragment of exon 2 [25] was inserted into the EcoR1 multiple cloning site of the pSVK3 plasmid vector (Pharmacia) in either the sense of antisense orientations relative to the SV40 origin and early promoter regions. The constructs were checked for fidelity by restriction enzyme mapping. The aminoglycoside phosphotransferase (neo) gene was then cloned into the Hpa1 restriction site of the same plasmid under the control of the SV40 early promoter.

Cell transfection was performed by electroporation using the ElectroPorator Apparatus (Invitrogen) at 200 V. The plasmid DNA (5 μg) was linearized at the PvuII restriction site and mixed with 10^6 cells suspended in 400 μl Hepes-buffered saline and kept on ice. The transfected cells were cultured in RPMI medium containing 5% FCS and antibiotics and supplemented from day 2 onward with 200 μg/ml G418 (Geneticin; Gibco). G418-resistant clones were isolated 2–3 weeks later.

2.3. RNA extraction and Northern blot analysis

Cell monolayers (at 80% confluency) were scrapped, centrifuged and the pellets lysed with an NP-40 lysis buffer (0.15 M NaCl containing 1 mM Tris-HCl, pH 7.5, 1.5 mM $MgC1_2$ and 0.5% NP-40)

for 5 min on ice. The lysate was cleared by centrifugation and the supernatant mixed with urea buffer (0.35 M NaC1 with 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 7 M urea and 1% SDS) and phenol/chloroform/isoamyl alcohol (50:48:2) and left overnight at −80°C. RNA was precipitated from the aqueous phase with absolute ethanol and stored at −20°C.

For Northern blot analysis, RNA (15 μg/lane) was separated on 1% agarose-formaldehyde gels, transferred onto Hybond-N nylon membranes (Amersham) by capillary action in 20 × SSC and covalently cross-linked by UV irradiation. Prehybridization and hybridization of the membranes were carried out at 42°C. The filters were hybridized either with a synthetic oligonucleotide probe, corresponding to bases 246–278 of type I collagenase cDNA [24] which was end-labeled with ^{32}P-ATP or with a 1.1 kb type IV collagenase cDNA fragment (kindly provided by Dr. W. Stetler-Stevenson, NIH) which was labeled by the random primer extension labeling system (DuPont) using ^{32}P-CTP. A ^{32}P-ATP end-labeled 18S oligonucleotide probe was used as a control for RNA loading. The hybridized filters were washed twice at 55°C for 30 min with either 6 × SSPE (filters probed with oligonucleotides) or 0.1 × SSC containing 0.1% SDS (filters probed with cDNA) and autoradiographed at −80°C. The relative amounts of mRNA transcripts were analysed by laser densitometry using an LKB Ultroscan XL Enhanced laser densitometer and normalized relative to the 18S rRNA controls.

2.4. Preparation of conditioned media

Cultures were grown to 80% confluency in 6-well plates, washed twice with serum-free RPMI and cultured in serum-free RPMI for 72 h. The supernatants were collected, cells and debris removed by centrifugation and filtration and the cleared supernatants dialyzed against a collagenase buffer (1 mM Tris-HCl, pH 7.6, with 1 mM $CaC1_2$) for 24 h, aliquoted, concentrated by freeze-drying at −120°C using a cooling trap (HETOTRAP CT 110) and stored at −20°C until used.

2.5. Western blot analysis

Western blot analysis was performed essentially as we described previously [26]. Briefly, serum-free conditioned media (50 × concentrated) were separated on a 10% SDS-polyacrylamide gel and the proteins electrophoretically transferred onto nitrocellulose filters (0.2 mm; Schleicher and Schuell). The blots were probed either with a mouse monoclonal antibody to MMP-1 (Cedarlane Laboratories, Hornby, Ontario) [27] or with a rabbit antiserum to MMP-2 (a kind gift from Dr. William Stetler-Stevenson, NIH). Alkaline phosphatase-conjugated affinity purified goat anti-mouse IgG or goat anti-rabbit IgG (Bio/Can Scientific, Mississauga, Ontario) were used as second antibodies, both at a dilution of 1:2000.

2.6. Zymography

The zymographic analysis was carried out essentially as we previously described [28]. SDS-polyacrylamide (10%) gels [29] were co-polymerized with gelatin or casein both at a final concentration of 1 mg/ml or with human placental type IV collagen at a concentration 0.4 mg/ml. The concentrated conditioned media were mixed 3:1 (v/v) with sample buffer (0.3 M Tris-HCl, pH 6.8, containing 8% SDS, 0.4% bromophenol blue and 40% glycerol), loaded onto the gels without boiling and separated by electrophoresis. The gels were washed for 1 h in a solution of 2.5% Triton X-100 in 40 mM Tris-HCl, pH 7.6, and for 15 min in 10 mM Tris-HCl, pH 8. For the enzymatic reaction to take place, the gels were incubated for 18 h at 37°C in a solution of 50 mM Tris-HCL, pH 8, containing 10 mM $CaC1_2$. The gels were stained for 2 h in a 0.5% Coomassie blue R250 solution, then destained in 20% methanol with 10% acetic acid until clear bands (indicating lysis) were apparent on the blue background. Prestained molecular weight markers were resolved on the same gels, separated from other samples after electrophoresis and fixed in 5% acetic acid. To characterize the protease bands, some of the gels were incubated in a buffer (50 mM Tris-HCl, pH 8, with 10 mM $CaC1_2$) containing 20 mM EDTA (a metalloproteinase inhibitor).

2.7. Invasion assays

The chemoinvasion assays were performed using 8 μm polycarbonate filters coated with reconstituted basement membrane (Matrigel; Collaborative Re-

search), type I (rat tail; Boehringher-Manneheim) or type IV (human placenta; Sigma) collagen. Prior to coating, Matrigel was diluted with cold distilled water and 13 μg in 60 μl were added to each filter. Type IV collagen was diluted in 0.1% acetic acid and used at concentrations of 40–400 μg per filter. Because of batch-to-batch variations, preliminary tests were performed for each batch of commercially obtained pepsin-extracted type IV collagen, and the collagen concentration readjusted to standardize the assay conditions in respect to the levels of invasion of control cells. Type I collagen was soaked overnight in 0.1% acetic acid, mixed with $10\times$ concentrated PBS and 0.1 N NaOH at a ratio of 8:1:1 and added to the filters at the indicated concentrations. The coated filters were dried overnight and equilibrated with serum-free RPMI for 2 h. The medium was then removed, the filters placed in 24-well plates and to each filter 5×10^4 cells were added in 100 μl RPMI containing 0.2% BSA for a 48 h incubation at 37°C, in a humidified 5% CO_2 incubator. To block metalloproteinase activity, the synthetic peptidylhydroxamate inhibitor U24522 [(R,S)-N-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl-L-leucyl-L-phenylalaninamide] [30] generously provided by Dr. G. Dipasquale (Stuart Pharmaceuticals, Wilmington Delaware) was added with the cells at concentrations of 40–80 μM which were predetermined to have no cytotoxic effect. Human fibronectin (5 μg/ml in RPMI) was used as a chemoattractant in the lower chamber. At the end of the incubation period, the cells on the upper surface of the filters were removed with a cotton swab and the filters fixed in 0.1% glutaraldehyde and stained with 0.2% crystal violet. The number of cells that migrated to the lower side of the filter was counted using a Nikon inverted microscope ($400\times$). A total of 20 random fields were counted per filter and 2 filters were used for each assay condition. Migration assays were carried out in a similar manner using filters which were pre-coated with a low concentration of type IV collagen (7.5 μg/filter) to facilitate cell spreading [28].

3. Results

To assess the role of metalloproteinases in melanoma MIM invasion, the cells were added to Matrigel-coated filters together with the synthetic metalloproteinase inhibitor U24522 at concentrations (40–80 μM) predetermined to have no cytotoxic effect on the cells. Results shown in Fig. 1 demonstrate that this inhibitor blocked cell invasion in a dose dependent manner by up to 55% and confirm the involvement of metalloproteinases in the invasion.

MIM cells constitutively express both MMP-1 and MMP-2. To analyse the role of MMP-1 in the invasion, MIM cells were transfected with a pSVK3 plasmid expressing a 777 bp MMP-1 insert in the sense or antisense orientations relative to the SV-40 promoter. Several clones were obtained and the extent of MMP-1 mRNA suppression was evaluated by the Northern blot assay. Two clones, 25-2 and 25-12, which had marked reductions in MMP-1 mRNA levels (Fig. 2, panel a) were selected for further analysis. The reductions in the MMP-1 mRNA transcripts in these clones as calculated by comparison to MIM wild-type cells and standardized relative to the 18S rRNA were 90% and 96% respectively. In these clones, as well as in the sense-transfected clone 7-1, the mRNA levels for MMP-2 were either unchanged or slightly higher than in the wild-type cells (Fig. 2, panel b).

To assess MMP-1 expression at the protein level, a Western blot analysis was performed. A single, M_r 48 000 band corresponding to the active form of

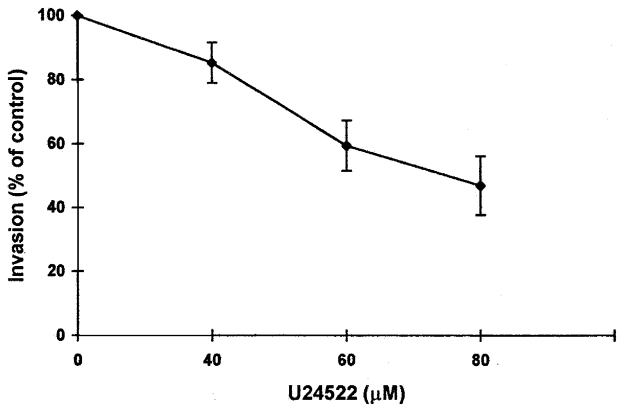

Fig. 1. Inhibition of MIM invasion by the metalloproteinase inhibitor U24522. MIM cells were added to the Matrigel-coated filters (5×10^4 cells/filter) with or without the indicated concentrations of the inhibitor. Invasion was analyzed following a 48 h incubation at 37°C. The results are expressed as the percent of invading cells relative to control MIM cells.

MMP-1 was seen in blots prepared from medium conditioned by wild-type MIM cells. This band could not be detected in parallel blots of conditioned media derived from either one of the transfectants, confirming that MMP-1 protein synthesis in these cells was also suppressed (Fig. 3).

Suppression of MMP-1 production was also confirmed at the functional level. When the ability of 25-12 cells to degrade type I collagen was measured using 8 μm filters precoated with graded concentrations of type I collagen, we found that the relative invasiveness of the transfectants decreased with in-

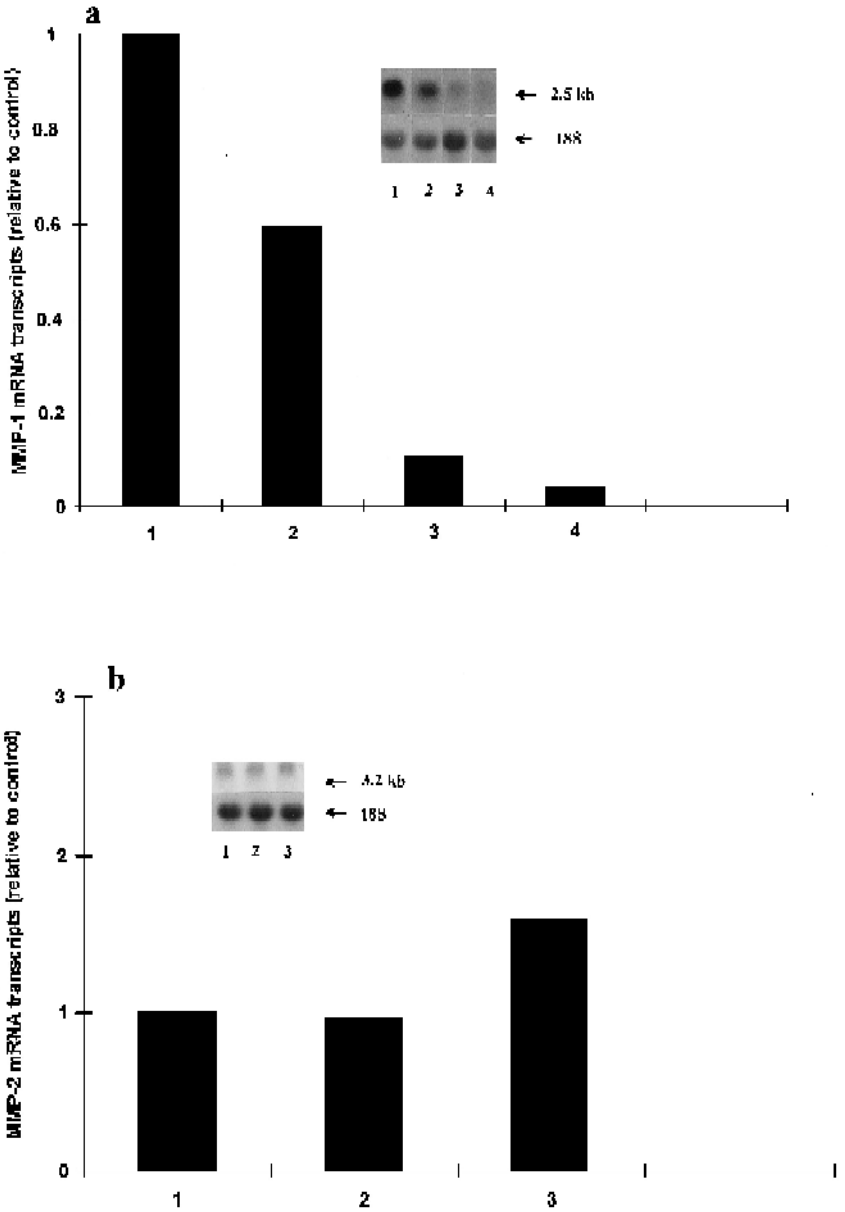

Fig. 2. Northern blot analysis of MMP-1 and MMP-2 mRNA in antisense-transfected cells. Fifteen μg of total RNA were loaded in each lane. a: The blots were hybridized first with an oligonucleotide probe specific for MMP-1 and then with an oligonucleotide probe for 18S rRNA. Lane 1: wilde-type MIM cells, Lane 2: sense-transfected clone 7-1, Lanes 3 and 4: antisense-transfected clones 25-2 and 25-12. b: The blots were hybridized successively with the 1.1 kb cDNA fragment of MMP-2 and an 18S rRNA-specific oligonucleotide probe. Lane 1: sense-transfected clone 7-1; Lanes 2 and 3: antisense-transfected clones 25-2 and 25-12. The results of the densitometry are shown in the bar graph. All values have been normalized relative to MIM wild-type cells which were assigned a value of 1.

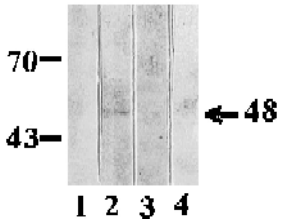

Fig. 3. Western blot analysis of MMP-1. Conditioned media derived from wild-type MIM (lanes 1,2), clone 25-12 (lane 3) and clone 25-2 (lane 4) cells were concentrated ($\times 50$) and the proteins (10 μg per lane) resolved on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were probed with a mouse monoclonal antibody to MMP-1 (lanes 2,3,4) and normal mouse serum (lane 1), as control. The positions of MW markers ($\times 10^{-3}$) are shown on the left. The position of the active form is indicated with an arrow on the right.

creasing concentrations of collagen reaching 20% of control levels at a concentration of 40 μg type I collagen (Fig. 4).

In order to analyse the expression and function of MMP-2 in the transfected cells, gel zymography and a Western blot analysis were subsequently performed.

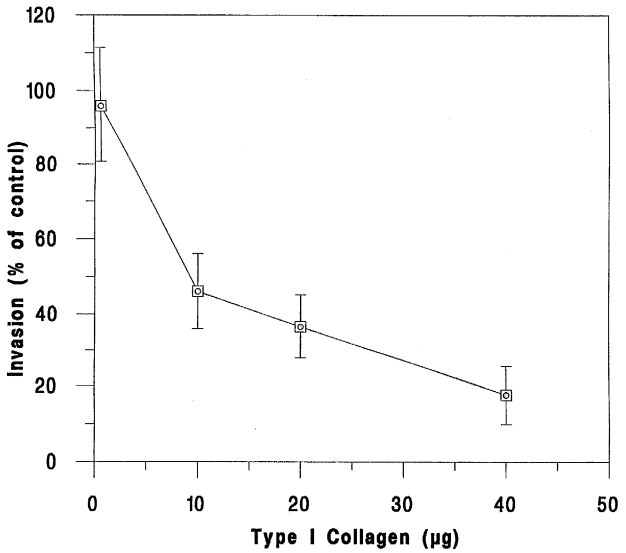

Fig. 4. Loss of type I collagen degradation and invasion in cells expressing MMP-1 antisense mRNA. Wild-type MIM and antisense transfected clone 25-12 cells were seeded onto filters (5×10^4 cells/filter) precoated with collagen at the indicated concentrations. Incubation was for 48 h at 37°C. Shown are means (and SD) of 3 experiments each performed in duplicates. Invasion of 25-12 cells was significantly lower than that of MIM cells ($P < 0.05$, $P < 0.025$ and $P < 0.01$ for 10, 20 and 40 μg type I collagen, respectively).

When serum-free conditioned media derived from MIM and transfected cells were analysed on polyacrylamide gels co-polymerized with 1 mg/ml gelatin, several bands of lysis were observed in all the zymograms (Fig. 5, panel a). These gelatinolytic activities were completely inhibited when the gels were incubated in the presence of 20 mM EDTA (not shown) indicating that they were mediated by metalloproteinases.

Two of the bands migrated in the M_r 66–72 000 region corresponding to the predicted molecular weights for MMP-2. These bands were of comparable intensities in all the zymograms suggesting that the functional levels of this gelatinase were unaltered.

When an anti-MMP-2 antibody was subsequently used to analyse conditioned media derived from clones 25-2 and 25-12 in a Western blot assay, a 66 kDa band corresponding to the active form of MMP-2 was detected (Fig. 6). Consistent with the zymography results (Fig. 5, panel a), the MMP-2 protein bands seen on Western blots of conditioned media derived from antisense-transfected and control cells were of comparable intensities.

Two weak gelatinolytic bands of 57 and 54 kDa were also observed in all the zymograms. This molecular mass range is characteristic of the proenzyme forms of MMP-1 and stromelysin [7,11,31]. However, these bands were not detectable in Western blots probed with an antibody to the C-terminal domain of MMP-1 [27], suggesting that they were probably due to stromelysin activity. Moreover,

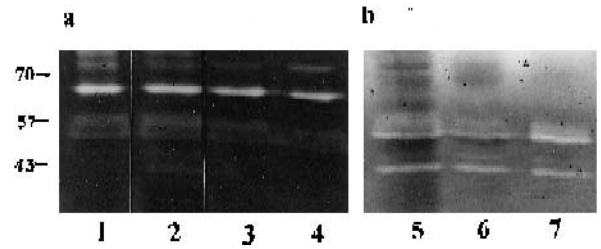

Fig. 5. Analysis of proteinase activity in MIM cells by gel zymography. Serum-free conditioned media derived from wild-type MIM (lanes 1,5), clone 7-1 (lane 2), clone 25-2 (lanes 3,7) and clone 25-12 (lanes 4,6) cells were concentrated ($\times 100$) and the proteins (25 μg/lane) resolved on 10% SDS-polyacrylamide gels which were co-polymerized with 1 mg/ml gelatin (panel a) or casein (panel b). The positions of MW markers ($\times 10^{-3}$) are shown on the left.

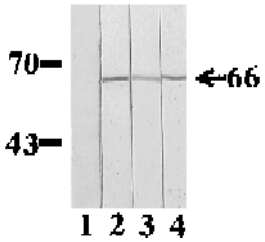

Fig. 6. Western blot analysis of MMP-2. Conditioned media derived from wild-type MIM (lanes 1,2), clone 25-12 (lane 3) and clone 25-2 (lane 4) cells were concentrated ($\times 50$) and the proteins resolved on 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters. The filters were probed with a rabbit antiserum to MMP-2 (lanes 2,3 and 4) or normal rabbit serum as control (lane 1) both at a dilution of 1:250. The positions of MW markers ($\times 10^{-3}$) are shown on the left. The position of the active intermediate is indicated with an arrow on right.

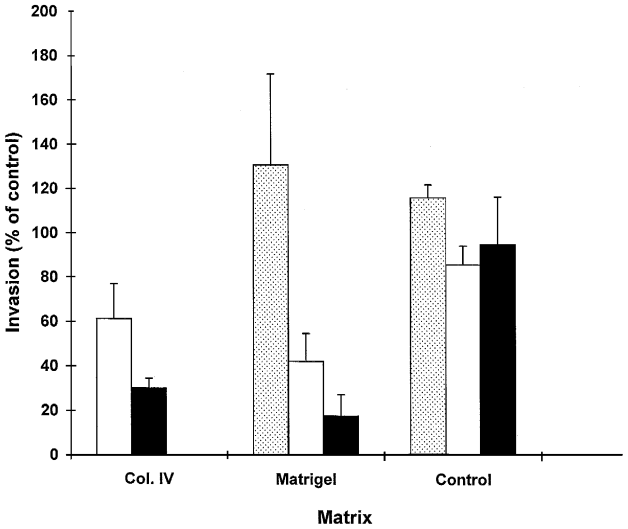

Fig. 7. Suppression of type IV collagen and Matrigel invasion in melanoma cells expressing MMP-1 antisense mRNA. Antisense transfected 25-2 (open bars) and 25-12 (solid bars) and sense transfected 7-1 (shaded bars) cells were seeded onto the coated filters at a density of 5×10^4 cells/filter. Invasion was measured following a 48 h incubation at 37°C. The results are expressed as percent of invading cells relative to control, wild-type MIM cells which were included in every experiment and represent means (and SD) of 2–3 experiments. The invasion of 25-2 and 25-12 cells was significantly lower than that of MIM cells on filters precoated with type IV collagen ($P < 0.05$ and $P < 0.005$) or Matrigel ($P < 0.01$ and $P < 0.0005$, respectively). Filters precoated with 7.5 μg type IV collagen were used as migration controls.

MMP-1 is known to have poor gelatinolytic activity [32,33] and stromelysin has been shown to produce more prominent zones of lysis on casein than on gelatin substrate gels [34]. When the intensities of these bands on both types of gels were compared, we found that the gelatinolytic activities associated with these weak zones of lysis resulted in more prominent casein degradation (Fig. 5, panel b), consistent with the conclusion that they were produced by stromelysin. The additional bands which became visible on the casein-containing gels (42–45 kDa, see Fig. 5, panel b) may represent the activated forms of stromelysin [7].

Subsequently, the invasive abilities of the transfectants were analysed using 8 μm filters precoated with type IV collagen or Matrigel. We observed a marked reduction in the abilities of 25-2 and 25-12 cells to invade through type IV collagen or Matrigel-coated filters as compared to control, wild-type cells (Fig. 7). Cell migratin as measured on uncoated filters (not shown) or on filters precoated with 7.5 μg/filter type IV collagen (Fig. 7) was comparable to that of control cells.

To assess type IV collagen degradation in a more direct manner, zymography was performed using polyacrylamide gels co-polymerized with placental type IV collagen. We found that conditioned medium derived from wild-type MIM cells produced a zone of lysis in the M_r 66–68 000 region corresponding to the predicted molecular weight range for activated MMP-2. This zone of lysis was either not apparent or considerably diminished in zymograms of conditioned media from clones 25-12 and 25-2 (Fig. 8).

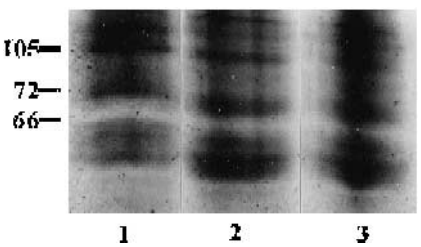

Fig. 8. Zymographic analysis of the type IV collagenolytic activity in MIM cells. Serum-free conditioned media derived from wild-type MIM (lane 1), clone 25-2 (lane 2) and clone 25-12 (lane 3) cells were concentrated ($\times 100$) and the proteins (20μg/lane) resolved on 10% SDS-polyacrylamide gels which were co-polymerized with 0.4 mg/ml placental type IV collagen. The positions of MW markers ($\times 10^{-3}$) are shown on the left.

4. Discussion

The dissolution of basement membrane barriers is a key event in cancer dissemination and metastasis. A number of proteinases have been implicated in tumor invasion [2,9,35] and their expression appears to vary in different tumor types [15,19,20,36]. In melanoma cells MMP-1, MMP-2 and MMP-9 were shown to be constitutively upregulated [19,33,37]. However, the relative contributions of these enzymes to melanoma invasion and metastasis are not fully understood.

In the present study, an invasive, human melanoma cell line, MIM, in which both MMP-1 and MMP-2 are constitutively expressed was used. To analyse the role of MMP-1 in invasion in this model, its expression was suppressed by stable transfection with a plasmid vector expressing an MMP-1 DNA fragment in the antisense orientation relative to the promoter. As expected, we found that melanoma cells in which MMP-1 mRNA levels were greatly reduced, lost their ability to invade through a matrix barrier composed of type I collagen. The surprising finding was that these cells also had a significantly reduced ability to invade through type IV collagen and a reconstituted basement membrane (Matrigel) in which type IV collagen is the major matrix constituent [38]. This reduction was seen despite the unaltered high production of MMP-2 by these cells as confirmed at both the mRNA and protein levels and despite the apparent expression of stromelysin-1, as suggested by the results of zymography with casein-containing gels.

We observed that the level of MMP-1 mRNA in the sense-transfected clone 7-1 cells was lower (40% reduction) than in the MIM wild-type cells. The reason for this difference is not clear. It may suggest that within the wild-type MIM cell line, clonal subpopulations express variable levels of this enzyme and the selected clone 7-1 was at the low end of this spectrum. Alternatively, this reduction may have resulted from the transfection and may possibly be related to the site of DNA integration. However, as the invasion of clone 7-1 cells on all the matrices tested was comparable to control MIM cells, it appears that this reduction in mRNA levels was not sufficient to significantly impact on the matrix-degrading ability of the cells.

Several mechanisms may be invoked in interpreting our data. MMP-1, whose main function is thought to be the dissolution of fibrillar type I and III collagens by cleaving at a site within the triple helix [2] has also been reported to degrade full length type IV collagen [32]. On the other hand MMP-2 and MMP-9 have been shown to degrade only pre-cleaved or conformationally altered forms of type IV collagen [39,40], prompting the suggestion that they may not be true type IV collagenases but may in fact act secondarily to other enzymes and mediate further degradation of an already processed type IV collagen [39]. It is possible, therefore, that in MIM cells, MMP-1 acts initially to degrade the native type IV collagen while the gelatinases only complete the degradative process. In cells which do not elaborate the required levels of MMP-1, this process may be blocked and the catalytic activity of the gelatinases may be insufficient to degrade the basement membrane structure. This interpretation is compatible with our observation that there was no apparent reduction in the gelatinolytic activities detected in conditioned media of the antisense-transfectants as measured by zymography, while the same cells had a significantly reduced ability to degrade type IV collagen or Matrigel. It is also in accord with our finding that a prominent zone of lysis corresponding to active forms of MMP-2 was present in type IV collagen zymograms obtained from medium conditioned by MIM cells but was diminished or absent in media conditioned by 25-2 or 25-12 cells.

Another possibility is that MMP-1 and MMP-2 may be involved in a proteolytic cascade where activation of the latter requires the catalytic action of the former [41] and the process is blocked when MMP-1 levels are sufficiently reduced. Proteolytic cascades involving members of the same or different families of matrix-degrading proteinases have been described in other tumor models [42]. However this mechanism may not be critical in the present model because the active, 66 kDa form of MMP-2 could be found in conditioned media derived from the antisense-transfectants. This suggests that MMP-2 activation could proceed in the absence of MMP-1, possibly through the proteolytic activity of other metalloproteinases [43,44].

We found that wild-type MIM cells and the transfectants produced low and comparable levels of TIMP-1 (data not shown) suggesting that suppression of MMP-1 transcription did not directly affect the

synthesis of the inhibitor. This raises the possibility that in the transfectants, the decrease in MMP-1 levels may have resulted in an increased level of unbound TIMP which could block the activity of other metalloproteinases (e.g., MMP-2). While we cannot entirely ruled out the possibility that a change in the TIMP:enzyme ratio had an effect on net proteolysis, two observations suggest that this was not a major mechanism: (1) a reduction in collagenolytic activity was seen in type IV collagen zymograms obtained with conditioned media from 25-2 and 25-12 cells (Fig. 8), despite the dissociation of TIMP and metalloproteinases under the assay conditions and b) antisense transfected clones in which the suppression of MMP-1 mRNA expression was only partial (50–70%), had unaltered levels of type IV collagen degradation and invasion (data not shown). The latter observation indicates that suppression of type IV collagen degradation was not proportional to the increase in the TIMP:metalloproteinase ratio and suggests that total inhibition of MMP-1 synthesis (such as was seen in clone 25-12] was required to achieve a significant suppression of type IV collagen degradation and invasion.

While the precise contribution of MMP-1 to type IV collagen degradation remains to be elucidated, our results suggest that the upregulated expression of this enzyme in vivo during the course of melanoma progression may not only provide the cells with a proteolytic mechanism for dissolution of dermal collagen during the vertical growth phase, but may also enhance basement membrane degradation which is required for blood vessel invasion and dissemination to distant organs. This enzyme may therefore provide an effective target for specific therapeutic intervention in the progression of melanoma metastasis.

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